

CLOINED AND ENGINEERED PLANTS AND METHOD OF USE FOR BIOREMEDIATION

This invention was made partially with government support awarded by the WV NASA Space Grant Consortium. The United States Government may have certain rights in the invention. This application claims benefit of priority to United States provisional patent application No. 60,131,513 to Rogers, filed April 29, 1999, which is incorporated herein by reference, and United States provisional patent application No. 60/171,127 to Rogers, filed December 16, 1999, which is incorporated by reference.

TECHNICAL FIELD

The present invention generally relates generally to the fields of plant molecular biology and bioremediation.

BACKGROUND

Cleanup of hazardous wastes by traditional technologies is projected to cost at least \$400 billion in the United States (Salt et al. (1995)). Of this amount, \$42.5 billion is the estimated cleanup costs for sites contaminated with heavy metals. Over the past century water polluted with heavy metals has become an increasing problem (Nriagu et al. (1988)). Contaminated sites leach large quantities of mercury and other heavy metals into water systems, resulting in thousands of square miles of contaminated wetlands, lakes, rivers and estuaries (Leigh (1994)). Heavy metal pollution problems are difficult and expensive to solve. Toxic organic compounds can be degraded enzymatically by microorganisms, while toxic metals cannot (Cunningham et al. (1995)). Heavy metals can only be remediated through evacuation to hazardous waste landfills or chemical leaching, remediation technologies that are quite expensive. Even microbial based remediation requires soil extraction. Phytoremediation, using wetland plants to remediate contaminated soils and waters, is a cost effective alternative to traditional cleanup methods.

Genes coding for certain remediation activities, such as remediation of heavy metals, such as mercury, have been identified (Heaton et al., 1998). Gene transfer methods offer the ability

of genetically altering certain plants for targeted remediation activities. For example certain plants have been engineered with a metallothionein gene to accumulate heavy metals.

However, genetically engineered wetland plants have not been used to perform bioremediation processes, probably due to the difficulty of producing genetically engineered wetland plant cells and then producing regenerated plants from these genetically engineered wetland plant cells. Of particular difficulty are methods of transforming these cells, forming callus from these transformed cells, and ultimately regenerating a plant, such as a transgenic plant, from these transformed cells or callus.

The present invention provides wetland plants, particularly wetland monocots that can express foreign genes, such as those having bioremediation capabilities, and methods of using them to perform new capacities, such as bioremediation processes for a wide variety of pollutants, including heavy metals, such as mercury. The present invention also provides related benefits as well.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A, FIG. 1B, FIG. 1C, FIG. 1D and FIG. 1E depict a variety of plasmids and constructs useful in the present invention. **FIG. 1A** depicts plasmid pBISN1. **FIG. 1B** depicts plasmid pCAS1. **FIG. 1C** depicts plasmid pE1120, having ocs-mas "super-promoter" in pBI101.1. **FIG. 1D** depicts plasmid pATC940. **FIG. 1E** depicts plasmid pAL77, which is constructed in pFC3 (see, Lonsdale et al., Plant Molecular Biology Reporter 13:342-345 (1995)), where P.Ubi refers to Maize ubiquitin promoter plus first intron from pAHC25 (see, Christensen et al., Plant Molecular Biology 18:675-689).

FIG. 2A, FIG. 2B, FIG. 2C, FIG. 2D and FIG. 2E depict the regeneration of *J. effusus* using methods of the present invention. **FIG. 2A** depicts shoot proliferation from *J. effusus* seedling explants on medium supplemented with cytokinins (5 milligrams/l) VA (left), 2iP (middle) and kinetin (right). Media pH were 3.8 (top row) and 7.8 (bottom row). Cultures were grown in standard 60 x 15 mm Petri plates and were photographed after eight weeks in culture media. **FIG. 2B** depicts eight week old regenerated shoots of *J. effusus* showing blackening at the base of the shoots. Magnification is 1.6X. **FIG. 2C** depicts rooting of *J. effusus* shoots cultured on rooting media (NAA 1 milligram/l) in 6.3 mm Magenta boxes. The treatments were

rooting medium alone (left), rooting medium with charcoal (middle), and rooting medium with citric acid and ascorbic acid (right). Cultures were photographed after five weeks on rooting medium. **FIG. 2D** depicts greenhouse established plants made using methods of the present invention, from left to right, *Scirpus polyphyllus* (one year old), *Juncus accuminatus* (six months old), *Typha angustifolia* (eight months old), and *Juncus effusus* (two years old). Black pots are 23 cm in diameter. **FIG. 2E** depicts greenhouse established *T. latifolia* made using methods of the present invention (two years old), in a 30 cm diameter pot.

FIG. 3A, FIG. 3B, FIG. 3C, FIG. 3D, FIG. 3E and FIG. 3F depict the regeneration of *T. angustifolia* using methods of the present invention. **FIG. 3A** depicts *Typha angustifolia* showing callus and somatic embryos after eight weeks in the dark on MS medium with picloram (5 mg/l). **FIG. 3B** depicts an enlarged view of a somatic embryo. **FIG. 3C** depicts starch right globular and **FIG. 3D** depicts bipolar somatic embryos on MS medium with picloram after ten weeks in culture. A blue filter was used in photographic embryos. **FIG. 3E** depicts germinating somatic embryos showing shoots and roots cultured in the light on MS medium with BA (5 mg/l). **FIG. 3F** depicts greenhouse established *T. angustifolia* plants made using methods of the present invention.

FIG. 4A and FIG. 4B depict the regeneration of *T. angustifolia* using methods of the present invention. **FIG. 4A** depicts germinating somatic embryos of *Typha angustifolia* cultured in the light on MS medium with GA (5 mg/l) at magnification 2.3X, **FIG. 4B** at 9.3X.

FIG. 5A, FIG. 5B, FIG. 5C, FIG. 5D, FIG. 5E, FIG. 5F, FIG. 5G and FIG. 5H depict the regeneration of *T. latifolia* using methods of the present invention. **FIG. 5A** depicts seeds of *Typha latifolia* (bar is 0.4 cm). **FIG. 5B** depicts three day old seedling explants used for callus induction (bar is 1 cm). **FIG. 5C** depicts callus induction from root shoot junction and from root of *Typha* seedling (bar is 0.15 cm). **FIG. 5D** depicts nine week old callus growing on MS medium supplemented with 5 mg/l of BA (bar is 1.5 cm). **FIG. 5E** depicts shoot regeneration from callus cultured on MS medium supplemented with 5 mg/l BA (bar is 0.5 cm). **FIG. 5F** depicts callus showing shoot and root initiation, on medium supplemented with 5 mg/l (bar is 0.2 cm). **FIG. 5G** depicts establishment of *in vitro* regenerated plants in the greenhouse made using methods of the present invention (photograph was taken one month after transfer to pots, bar is 12 cm). **FIG. 5H** depicts greenhouse established plants made using methods of the present

invention showing high root mass (photograph was taken three months after transfer to pots, bar is seven sm).

FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 6E, FIG. 6F, FIG. 6G and FIG. 6H depict the regeneration of *T. latifolia* and *T. angustifolia* using methods of the present invention. **FIG. 6A** depicts three day old seedling explants of *T. latifolia* used for callus induction at magnification 1.25X. **FIG. 6B** depicts dark grown nine week old callus of *T. latifolia* on P5 medium at magnification 1.4X. **FIG. 6C** depicts shoot regeneration from *T. latifolia* callus cultured on B5 medium at magnification 2.0X. **FIG. 6D** depicts eight week old callus of *T. angustifolia* cultured in the light on P5 medium showing somatic embryos at magnification 21.7X. **FIG. 6E** depicts *T. angustifolia* callus (top) and somatic embryos (bottom) stained with I2-K where starch rich somatic embryos showing dark blue color, whereas callus did not stain for starch at magnification 20.2X. **FIG. 6F** depicts five week old calli of *T. latifolia* (top) and *T. angustifolia* (bottom) transformed with Agrobacterium containing pBISN1 where blue color was developed with a GUS assay, magnification 3X. **FIG. 6G and FIG. 6H** depict close up views of x-gluc stained calli of *T. latifolia* (left, magnification 5.0X) and *T. angustifolia* (right, magnification 13.3X).

FIG. 7A, FIG. 7B, FIG. 7C, FIG. 7D and FIG. 7E depict (**FIG. 7A**) generation of *J. effusus* shoots, (**FIG. 7B**) *T. angustifolia* germinating somatic embryo, (**FIG. 7C**) regenerated *J. accuminatus* greenhouse plants, (**FIG. 7D**) regenerated *J. effusus* in constructed wetland (**FIG. 7E**) and transformed *T. latifolia* calli, using methods of the present invention.

FIG. 8 depicts regenerated plants, made using methods of the present invention, established in a constructed wetland. (A) *T. angustifolia*, (B) *J. effusus*, (C) *J. accuminatus*, (D) *C. lurida* and (E) *S. polyphyllus*.

FIG. 9A, FIG. 9B. and FIG. 9C depicts regenerated *J. effusus* using methods of the present invention.

SUMMARY

The present invention recognizes that plant cells, particularly plant cells from freshwater monocot plants, can be transformed and regenerated, particularly to produce plants that have bioremediative capacities.

A first aspect of the present invention is a method for transforming a plant cell, preferably a freshwater monocot plant cell such as the freshwater emergent wetland monocots *Carex*, *Scirpus*, *Juncus* or *Typha*. This aspect of the present invention includes cells and populations of cells, including callus, plants and seeds, made by or derived from this method.

A second aspect of the present invention is a method for transforming a plant cell using homologous recombination. Preferably, the plant cell to be transformed has a nucleic acid sequence that does not naturally occur in the plant cell that can be expressed by the plant cell, such as a reporter gene. A nucleic acid sequence of interest can be targeted to integrate into the locus of such nucleic acid sequence that does not naturally occur in the plant cell using homologous recombination. The nucleic acid sequence of interest can include any gene, but preferably includes a bioremediation gene.

A third aspect of the present invention is a method for regenerating a plant, such as a freshwater monocot plant, preferably a *Carex*, *Scirpus*, *Juncus* or *Typha*, preferably *Juncus effusus*, *Juncus accuminatus*, *Carex lurida* or *Scirpus polyphyllus*. The method includes providing a sample of a plant, inducing shoot development from the sample, and inducing root development from the sample. This aspect of the present invention includes plants made by this method and seeds derived therefrom.

A fourth aspect of the present invention is a method for regenerating a plant, such as a freshwater monocot plant, preferably a *Carex*, *Scirpus*, *Juncus* or *Typha*, preferably *Typha latifolia* or *Typha angustifolia*. The method includes providing a sample of a plant, forming a callus from the sample, inducing shoot development and inducing root development from the sample. This method can result in the formation of somatic embryos as well as callus. This aspect of the present invention includes plants made by this method and seeds derived therefrom.

A fifth aspect of the present invention is a method for regenerating a plant, such as a freshwater monocot plant, preferably a *Carex*, *Scirpus*, *Juncus* or *Typha*, preferably *Juncus accuminatus*. The method includes providing a sample of a plant, forming a callus from said

sample, inducing shoot development from the sample and inducing root development from the sample. This aspect of the present invention includes plants made by this method and seeds derived therefrom.

A sixth aspect of the present invention is a method for regenerating a plant or forming a somatic embryo from a plant, such as a freshwater monocot plant, preferably a *Carex*, *Scirpus*, *Juncus* or *Typha*, preferably *Typha angustifolia*. The method includes providing a sample of a plant, forming a callus from said sample, and inducing somatic embryogenesis from the sample. This aspect of the present invention includes plants made by this method and seeds derived therefrom.

A seventh aspect of the present invention is a method of bioremediation by exposing a plant made by a method of the present invention to an environment or sample that contains or is suspected of containing at least one contaminant that can be reduced by the plant.

DETAILED DESCRIPTION OF THE INVENTION**DEFINITIONS**

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, chemistry, microbiology, molecular biology, cell science and cell culture described below are well known and commonly employed in the art. Conventional methods are used for these procedures, such as those provided in the art and various general references (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989)). Where a term is provided in the singular, the inventors also contemplate the plural of that term. The nomenclature used herein and the laboratory procedures described below are those well known and commonly employed in the art. As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

“Isolated polynucleotide” refers to a polynucleotide of genomic, cDNA, or synthetic origin, or some combination thereof, which by virtue of its origin, the isolated polynucleotide (1) is not associated with the cell in which the isolated polynucleotide is found in nature, or (2) is operably linked to a polynucleotide that it is not linked to in nature. The isolated polynucleotide can optionally be linked to promoters, enhancers, or other regulatory sequences.

“Isolated protein” refers to a protein of cDNA, recombinant RNA, or synthetic origin, or some combination thereof, which by virtue of its origin the isolated protein (1) is not associated with proteins normally found within nature, or (2) is isolated from the cell in which it normally occurs, or (3) is isolated free of other proteins from the same cellular source, for example, free of cellular proteins), or (4) is expressed by a cell from a different species, or (5) does not occur in nature.

“Polypeptide” is used herein as a generic term to refer to a native or non-native protein, fragments thereof, or analogs of a polypeptide sequence.

“Active fragment” refers to a fragment of a parent molecule, such as an organic molecule, nucleic acid molecule, or protein or polypeptide, or combinations thereof, that retains at least one activity of the parent molecule.

“Naturally occurring” refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism, including viruses, that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally occurring.

“Operably linked” refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence operably linked to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

“Control sequences” refer to polynucleotide sequences that effect the expression of coding and non-coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequences; in eukaryotes, generally, such control sequences include promoters and transcription termination sequences. The term control sequences is intended to include components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

“Polynucleotide” refers to a polymeric form of nucleotides of a least ten bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA or RNA.

“Genomic polynucleotide” refers to a portion of the genome.

“Active genomic polynucleotide” or “active portion of a genome” refer to regions of a genome that can be up regulated, down regulated or both, either directly or indirectly, by a biological process.

“Directly” in the context of a biological process or processes, refers to direct causation of a process that does not require intermediate steps, usually caused by one molecule contacting or binding to another molecule (the same type or different type of molecule). For example, molecule A contacts molecule B, which causes molecule B to exert effect X that is part of a biological process.

“Indirectly” in the context of a biological process or processes, refers to indirect causation that requires intermediate steps, usually caused by two or more direct steps. For example, molecule A contacts molecule B to exert effect X which in turn causes effect Y.

“Sequence homology” refers to the proportion of base matches between two nucleic acid sequences or the proportion of amino acid matches between two amino acid sequences. When sequence homology is expressed as a percentage, for example 50%, the percentage denotes the proportion of matches of the length of sequences from a desired sequence that is compared to some other sequence. Gaps (in either of the two sequences) are permitted to maximize matching; gap lengths of 15 bases or less are usually used, 6 bases or less are preferred with 2 bases or less more preferred. When using oligonucleotides as probes or treatments, the sequence homology between the target nucleic acid and the oligonucleotide sequence is generally not less than 17 target base matches out of 20 possible oligonucleotide base pair matches (85%); preferably not less than 9 matches out of 10 possible base pair matches (90%), and most preferably not less than 19 matches out of 20 possible base pair matches (95%).

“Selectively hybridize” refers to sequences that detectably and specifically bind. Polynucleotides, oligonucleotides and fragments thereof selectively hybridize to target nucleic acid strands, under hybridization and wash conditions that minimize appreciable amounts of detectable binding to nonspecific nucleic acids. High stringency conditions can be used to achieve selective hybridization conditions as known in the art. Generally, the nucleic acid sequence homology between the polynucleotides, oligonucleotides, and fragments thereof and a nucleic acid sequence of interest will be at least 30%, and more typically and preferably of at least 40%. 50%, 60%, 70%, 80% or 90%.

Hybridization and washing conditions are typically performed at high stringency according to conventional hybridization procedures. Positive clones are isolated and sequenced. For example, a full length polynucleotide sequence can be labeled and used as a hybridization probe to isolate genomic clones from an appropriate target library as they are known in the art. Typical hybridization conditions and methods for screening plaque lifts and other purposes are known in the art (Benton and Davis, Science 196:180 (1978); Sambrook et al., supra, (1989)).

Two amino acid sequences are homologous if there is a partial or complete identity between their sequences. For example, 85% homology means that 85% of the amino acids are

identical when the two sequences are aligned for maximum matching. Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less are preferred with 2 or less being more preferred. Alternatively and preferably, two protein sequences (or polypeptide sequences derived from them of at least 30 amino acids in length) are homologous, as this term is used herein, if they have an alignment score of at least 5 (in standard deviation units) using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater (Dayhoff, in Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, volume 5, pp. 101-110 (1972) and Supplement 2, pp. 1-10). The two sequences or parts thereof are more preferably homologous if their amino acids are greater than or equal to 30% identical when optimally aligned using the ALIGN program.

"Corresponds to" refers to a polynucleotide sequence that is homologous (for example is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to all or a portion of a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence TATAC corresponds to a reference sequence TATAC and is complementary to a reference sequence GTATA.

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence," "comparison window," "sequence identity," "percentage of sequence identity," and "substantial identity." A reference sequence is a defined sequence used as a basis for a sequence comparison; a reference sequence can be a subset of a larger sequence, for example, as a segment of a full length cDNA or gene sequence given in a sequence listing, or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides can each (1) comprise a sequence (for example a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A comparison window, as used herein, refers to a conceptual

segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window can comprise additions and deletions (for example, gaps) of 20 percent or less as compared to the reference sequence (which would not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window can be conducted by the local homology algorithm (Smith and Waterman, Adv. Appl. Math., 2:482 (1981)), by the homology alignment algorithm (Needleman and Wunsch, J. Mol. Bio., 48:443 (1970)), by the search for similarity method (Pearson and Lipman, Proc. Natl. Acid. Sci. U.S.A. 85:2444 (1988)), by the computerized implementations of these algorithms such as GAP, BESTFIT, FASTA and TFASTA (Wisconsin Genetics Software Page Release 7.0, Genetics Computer Group, Madison, WI), or by inspection. Preferably, the best alignment (for example, the result having the highest percentage of homology over the comparison window) generated by the various methods is selected.

“Sequence identity” means that two polynucleotide sequences are identical (for example, on a nucleotide-by-nucleotide basis) over the window of comparison.

“Percentage of sequence identity” is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (for example, the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

“Substantial identity” as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 30 percent sequence identity, preferably at least 50 to 60 percent sequence identity, more preferably usually at least 60 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25 to 50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence that may include deletions or additions that total 20 percent or less of the reference sequence over the window of comparison.

“Substantial identity” as applied to polypeptides herein means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 30 percent sequence identity, preferably at least 40 percent sequence identity, and more preferably at least 50 percent sequence identity, and most preferably at least 60 percent sequence identity. Preferably, residue positions that are not identical differ by conservative amino acid substitutions.

“Conservative amino acid substitutions” refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine and tryptophan; a group of amino acids having basic side chains is lysine, arginine and histidine; a group of amino acids having acidic side chains is aspartate and glutamate; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acid substitution groups are: valine-leucine-isoleucine; phenylalanine-tyrosine; lysine-arginine; alanine-valine; glutamate-aspartate; and asparagine-glutamine.

“Label” or “labeled” refers to incorporation of a detectable marker, for example by incorporation of a radiolabeled compound or attachment to a polypeptide of moieties such as biotin that can be detected by the binding of a section moiety, such as marked avidin. Various methods of labeling polypeptide, nucleic acids, carbohydrates, and other biological or organic molecules are known in the art. Such labels can have a variety of readouts, such as radioactivity, fluorescence, color, chemiluminescence or other readouts known in the art or later developed. The readouts can be based on enzymatic activity, such as that of beta-galactosidase, beta-lactamase, horseradish peroxidase, alkaline phosphatase or luciferase; or radioactive emissions from isotopes such as ^3H , ^{14}C , ^{35}S , ^{125}I or ^{131}I ; fluorescence from fluorescent proteins, such as green fluorescent proteins; or from other fluorescent labels, such as FITC, rhodamine, and lanthanides. Where appropriate, these labels can be the product of the expression of reporter genes, as that term is understood in the art. Examples of reporter genes are beta-lactamase (U.S. Patent No. 5,741,657 to Tsien et al., issued April 21, 1998) and green fluorescent protein (U.S. Patent No. 5,777,079

to Tsien et al., issued July 7, 1998; U.S. Patent No. 5,804,387 to Cormack et al., issued September 8, 1998).

“Substantially pure” refers to an object species or activity that is the predominant species or activity present (for example on a molar basis it is more abundant than any other individual species or activities in the composition) and preferably a substantially purified fraction is a composition wherein the object species or activity comprises at least about 50 percent (on a molar, weight or activity basis) of all macromolecules or activities present. Generally, as substantially pure composition will comprise more than about 80 percent of all macromolecular species or activities present in a composition, more preferably more than about 85%, 90%, 95% and 99%. Most preferably, the object species or activity is purified to essential homogeneity. (wherein contaminant species or activities cannot be detected by conventional detection methods) wherein the composition consists essentially of a single macromolecular species or activity. The inventors recognize that an activity may be caused, directly or indirectly, by a single species or a plurality of species within a composition, particularly with extracts.

“Sample” means a biological sample, such as a sample derived from a plant. A plant sample can be a whole plant at any stage of development (such as a mature plant, seedling, or the like), a portion of a plant, such as a sample of tissue from at least one portion of at least one plant (such as, for example, leaf, stem or roots), a callus, an immature inflorescence, a seed, an embryo, or a single cell or population of cells, or any combination thereof. Such samples can be obtained using methods known in the art, such as, for example, clipping, culturing, maceration, digestion or other methods.

“Population of cells” means more than one cell. The cells in a population cells can be the same or different. can be a mixture of cells from the same or different organism, and can be a mixture of prokaryotic and eukaryotic cells, such as a mixture of bacteria and plant cells. A population of plant cells can be in the form of dispersed individual cells, in the form of a callus, immature inflorescence, tissue samples from a plant or a portion thereof, samples of embryos or seeds, germinated seedlings, *in vitro* germinated seedlings, or any combination thereof.

“Freshwater wetland monocot plant” means a monocotyledonous plant that can grow in freshwater saturated soils and is preferably a vascular hydrophyte (Mitsch et al., 1993).

"Freshwater emergent wetland monocot plant" means a monocotyledonous plant rooted in soil with part of the plant that emerges from the water, that can grow in freshwater saturated soils and is a vascular phytrophyte (Mitsch et al., 1993).

"*Carex*" includes all members of that genus.

"*Juncus*" includes all members of that genus.

5 "Scirpus" includes all members of that genus.

"*Typha*" includes all members of that genus.

"Disarmed *Agrobacterium*" means an *Agrobacterium* that does not have the activity of forming tumors in plants. Examples of disarmed *Agrobacterium*, such as *Agrobacterium tumefactor*, include EHA105 and A281 (Hood et al., 1986) and AT789 and AT793 (Narasimhulu et al.).

10 "Vector" includes a nucleic acid molecule that is in a configuration (such as a plasmid) and/or packaged (such as in a virus or bacteria) such that at least a portion of the nucleic acid molecule can transfect or transduce a plant cell. A vector can include expression control sequences that are operable in the plant cell. A vector can include a nucleic acid molecule that encodes a gene of interest separate from the expression control sequence.

15 "Gene of interest" means any nucleic acid molecule that encodes a protein, peptide or polypeptide that has or is believed to have a biological activity, such as bioremediation, therapeutics, or any other biological activity. A gene of interest can be a bioremediation gene and can optionally be operably linked to expression control sequences, wherein such expression control sequences are preferably operable in a plant into which the gene of interest is to be inserted by, for example, transfection or transduction. A gene of interest can also encode an antisense molecule that has or is believed to have a biological activity.

20 "Flanking nucleic acid sequences" means nucleic acid sequences that are provided on the 3' or 5' end, or both, of a nucleic acid sequence, such as a nucleic acid sequence of interest. Flanking nucleic acid sequences can be attached to a nucleic acid sequence of interest using established methods, such as recombinant methods as they are known in the art.

25 "Reporter gene" means a gene that encodes a polynucleotide or polypeptide that is directly or indirectly detectable. For example, a reporter gene can be a fluorescent protein such as green fluorescent protein that is directly detectable. A reporter gene can be an enzyme whose presence

is detected by the conversion of a substrate, such as Xgluc, horseradish peroxidase, beta-lactamase or beta-galactosidase. Appropriate substrates to visualize the presence of such enzymes are readily available.

“Bioremediation gene” is a nucleic acid molecule that encodes a polypeptide or protein that has an activity related to bioremediation. Bioremediation gene nucleic acid sequences from an organisms other than a plant can be modified such that they contain codons preferred by a plant using methods known in the art. Bioremediation genes can also be present on operons. Bioremediation genes include, but are not limited to, genes affecting heavy metal bioremediation relating to contaminants such as copper, mercury, gold, cadmium, lead, telurite and silver such as merA, merB, merApe9, merApe20, merApe29, merApe38, merApe47, merT, merP (see, U.S. Patent No. 5,668,294, to Meagher et al., issued September 16, 1997; O’Gara et al., Appl. Environ. Microbiol. 63:4713-4720 (1997); Chen et al., Appl. Environ. Microbiol. 63:2442-2445 (1997); Jeffrey et al., Microb. Ecol. 32:293-303 (1996); Selifonova et al., Appl. Environ. Microbiol. 60:3503-3507 (1994)); chlorobenzoate, chlorobenzene, toluene and naphthalene such as Tn5271, Tn5542, Tn4653, Tn4651, Tn4656 and Tn4655 (see, Tan, Appl. Microbiol. Biotechnol. 51:1-12 (1999)); metallothionein such as HMT1A (see, Kotrba et al., J. Recept. Signal Transduct Res. 19:703-715 (1999)); nitriles such as Nhase (see, Kobayashi et al., Nat. Biotechnol. 16:733-736 (1998)); reduction and oxidation such as cytochromes, such as cytochrome c and P450 (see, Shimoji et al., Biochemistry, 37:8848-8852 (1998); Aubert et al., Appl. Environ. Microbiol. 64:1308-1312 (1998); Shanker et al., Bioetchnol. Prog. 12:474-479 (1996)); 2,4-dichlorophenoxyacetic acid such as tfd (see, Top et al. Antonie Van Leeuwenhoek 73:87-94 (1998); Sinclair, Appl. Environ. Microbiol. 60:4053-4058 (1994)); phenol such as phenomonooxygenase such as pheA (see, Peters et al., Appl. Environ. Microbiol. 63:4899-4906 (1997)); polycyclic aromatic hydrocarbons such as MnP (see, Bogan et al., Appl. Environ. Microbiol. 62:2381-2386 (1996)); toluene, napthalene, dodecane, hexadecane such as alkB, ndoB, todCl and xylE (see, Whyte et al., Can. J. Microbiol. 42:99-106 (1996)); polychorinated biphenyls such as bphABC (see, Damaj et al., Biochem. Biophys. Res. Commun. 26:908-915 (1996)); jet fuel such as nahAc, alkB and xylE (see, Chandler et al. Appl. Biochem. Biotechnol. 57-58:971-982 (1996)); chlorobenzoate such as cbaAB, Tn5271, IncP (see, Nakatsu et al., Mol. Ecol. 4:593-603 (1995)); trichloroethylene (see, Matin et al. Appl. Environ. Microbiol. 61:3323-3328 (1995));

alkylbenzoates such as Plac (see, Ronchel et al., Appl. Environ. Microbiol. 61:2990-2994 (1995)); trichloroethylene, polychlorinated biphenyls and polynuclear aromatic hydrocarbons (see, Sayler et al., Appl. Biochem. Biotechnol. 54:277-290 (1995)); biphenyls such as bph, TnPCB, F113pcb (see, Brazil et al., Appl. Environ. Microbiol. 61:1946-1952 (1995)); polychlorinated biphenyls such as IncP, pSS50 and pSS60 (see, Springael et al., Biodegradation 5:343-357 (1994)); polychlorinated biphenyls and 2,3-dihydroxybiphenyl and 2-chlorobiphenyl (see, Layton et al., K. Ind. Microbiol. 13:392-401 (1994)); alkylbenzoates, halobenzoates, halotolunes, salicylates such as xylS, xylR, nahR, xylS2 (see, de Lorenzo et al., Gene 130:41-46 (1993)); and polychlorinated biphenyls such as bphABC (see, Lajoie et al., Appl. Environ. Microbiol. 59:1735-1741 (1993)).

“Regeneration” in the context of regenerating a plant, means regenerating a whole plant, preferably a plant with reproductive capability, from, for example, a portion of a plant, a seedling, an embryo, a callus or a plant cell or a population of plant cells.

“Plant growth regulator” means a chemical or biochemical, such as a polypeptide or small molecule, that acts as a plant growth regulator or regulates plant development and differentiation for the purposes of the present invention. A plant growth regulator can be an auxin, such as, but not limited to, naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6-trichloropicolinic acid (picloram). A plant growth regulator can be a cytokinin, such as, but not limited to, N6-benzyladenic (BA), N6-(2-isopentenyl)-adenine (2iP), 1-phenyl-3-(1,2,3-thiodiazol-5-yl)-urea (thidiazuron) and 6-furfurylaminopurine (kinetin). Other plant growth regulators as they are known in the art or later developed, such as dicamba, are included as plant growth regulators.

“Inducing shoot development” means providing culture conditions, such as nutrients, plant growth regulators, light, temperature or minerals such that a plant sample produces shoot structures.

“Inducing root development” means providing culture conditions, such as nutrients, plant growth regulators, light, temperature or minerals such that a plant sample produces root structures.

“Inducing callus formation” or “forming a callus” means providing culture conditions, such as nutrients, plant growth regulators, light, temperature or minerals such that a plant sample produces a callus.

[illegible]

INTRODUCTION

The present invention recognizes that plant cells, particularly plant cells from freshwater monocot plants, can be transformed and regenerated, particularly to produce plants that have bioremediative capacities.

As a non-limiting introduction to the breath of the present invention, the present invention includes several general and useful aspects, including:

- 1) a method for transforming a plant cell, preferably a freshwater monocot plant cell such as the freshwater emergent wetland monocots *Carex*, *Scirpus*, *Juncus* or *Typha*;
- 2) a method for transforming a plant cell using homologous recombination;
- 3) a method for regenerating a plant, such as a freshwater monocot plant, preferably a *Carex*, *Scirpus*, *Juncus* or *Typha*, preferably *Juncus effusus*, *Carex lurida* or *Scirpus polyphyllus*;
- 4) a method for regenerating a plant, such as a freshwater monocot plant, preferably a *Carex*, *Scirpus*, *Juncus* or *Typha*, preferably *Typha latifolia*;
- 5) a method for regenerating a plant, such as a freshwater monocot plant, preferably a *Carex*, *Scirpus*, *Juncus* or *Typha*, preferably *Juncus accuminatus*;
- 6) a method for regenerating a plant or forming a somatic embryo from a plant, preferably a *Carex*, *Scirpus*, *Juncus* or *Typha*, preferably *Typha angustifolia*; and
- 7) a method of bioremediation by exposing a plant made by a method of the present invention to an environment or sample that contains or is suspected of containing at least one contaminant that can be reduced by the plant.

These aspects of the invention, as well as others described herein, can be achieved by using the methods, articles of manufacture and compositions of matter described herein. To gain a full appreciation of the scope of the present invention, it will be further recognized that various aspects of the present invention can be combined to make desirable embodiments of the invention.

I METHOD OF TRANSFORMING A PLANT CELL USING *AGROBACTERIUM*

The present invention includes a method for transforming a plant cell, preferably a freshwater monocot plant cell such as the freshwater emergent wetland monocots *Carex*, *Scirpus*, *Juncus* or *Typha*. This aspect of the present invention includes cells and populations of cells, including callus, plants and seeds, made by or derived from this method. The method for making a transformed a plant cell includes providing at least one cell from a plant, and inoculating a plant cell with at least one *Agrobacterium*.

Plants

Plants useful in the present invention include freshwater monocot plants, freshwater wetland monocot plants and freshwater emergent wetland monocot plants. Preferably, the plant is a member of the genus *Carex*, *Scirpus*, *Juncus* or *Typha* and more preferably is *Juncus effusus*, *Juncus accuminatus*, *Carex lurida*, *Typha latifolia*, *Typha angustifolia* or *Scirpus polyphyllus*. The cell used in this method can be any cell from a plant, such as a single cell, a population of cells, an immature inflorescence, a sample of callus, a portion of a plant, a seedling a seed or an embryo. Plant cells can be obtained using methods known in the art for a particular type of sample, such as cutting to obtain portions of plants.

Agrobacterium

The *Agrobacterium* used in the present invention is preferably a disarmed *Agrobacterium*, such a *Agrobacterium tumefaciens*, such that the *Agrobacterium* does not induce tumor formation in the plant cells. Preferably, the genes responsible or related to such tumor formation are inactive or removed from the *Agrobacterium*. Preferably, the *Agrobacterium* contains nucleic acid sequences that become integrated in the plant cell's genome such that the plant cell becomes stably transfected. Alternatively, nucleic acid sequences from the *Agrobacterium* can remain extrachromosomal as to the plant cell's genome, whereby the plant cell can be transiently or stably transfected.

Vectors

The *Agrobacterium* used in this method preferably contains a vector, such as those exemplified in the FIG. 1A through FIG. 1E and the Examples. Preferably the vector contains a gene of interest and expression control sequences that drive the expression of the gene of interest. In certain aspects of the present invention, *Agrobacterium* need not be used to introduce

a vector into a plant cell, such as when other methods, such as microballistics and lipofection can be used. Preferably, the gene of interest contains nucleic acid sequences that include codons that are preferred by the plant and the expression control sequences are operative in the plant. The expression control sequences can be chosen such that they drive the expression of the gene of interest in certain tissues, such as root, stems or leaves, or under certain conditions, such as nutrient deprivation. The vector can also include splice donor and/or splice acceptor sites, such that the vector preferably becomes integrated into the plant cell's genome (random integration or directed integration) but that need not be the case (see, WO 98/13353 to Whitney et al., published April 2, 1999; U.S. Patent No. 5,298,429 to Evans et al., issued March 29, 1994); Skarnes et al., Genes and Development 6:903-918 (1992); Reddy et al., Proc. Natl. Acad. Sci. USA 89:6721-6725 (1992); Kuspa et al., Proc. Natl. Acad. Sci. USA 89:8803-8807 (1992); Reid et al., Mol. Cell. Biol. 11:2769-2777 (1991)). The vector can also contain flanking nucleic acid sequences that can promote the integration of the vector into the plant cell's genome via homologous recombination (see, WO 96/30540 to Smith et al., published October 3, 1996).

The gene of interest can be any gene, preferably a reporter gene such as, but not limited to, *uidA* or GUS as in the plasmid vectors depicted in FIG. 1A through FIG. 1C or *merA* (FIG. 1E), or a bioremediation gene or genes. In one aspect of the present invention, a bioremediation gene can be fused to a DNA regulatory sequence of interest such as the ocs-mas promoter pATC940 (FIG. 1D). The gene of interest can also preferably be a bioremediation gene. Preferably, the gene of interest does not naturally occur in the plant, but that need not be the case. More than one gene of interest can be provided in a vector. For example, a vector can include a reporter gene operably linked to a bioremediation gene such that both are expressed in the transformed cell. The expression of the reporter gene and/or the bioremediation gene can be driven by expression control sequences on the plasmid or in the plant cell's genome. In this instance, the reporter gene serves the dual purpose of reporting that the vector is operating in the cell, and also reports that the bioremediation gene is also presumptively being expressed in the cell. That the bioremediation gene is being expressed and is active can be determined using methods known in the art, such as Northern blotting for the mRNA derived from the bioremediation gene and assaying for the activity of the protein encoded by the bioremediation gene.

In addition, instead of using *Agrobacterium* to deliver a vector to a plant cell, other methods known in the art can also be used. For example microballistics and electroporation can also be used for this purpose.

Cells, Callus, Somatic Embryos, Plants and Seeds

The present invention includes cells, populations of cells, callus, somatic embryos, plants and seeds made by a method of the present invention. Transformed cells made by the present invention can be cultured to form populations of cells using methods known in the art. In particular, the population of cells can be a callus. A population of cells can be of a single clonal population, or a combination of two or more cell types, such as transformed and non-transformed cells. Plants can be regenerated from cells made using the present invention using culturing methods, particularly those taught herein. Mature plants derived from the methods of the present invention can also be used to produce seeds, which are part of the present invention as well. Preferably, the cells, population of cells, callus, immature inflorescence, somatic embryos, plants and seeds either express the gene of interest or are capable of expressing the gene of interest under a set of conditions. Not all cells of a population of cells, callus, immature inflorescence, somatic embryos, plant or seed need express the gene of interest. For example, tissue expression patterns in a plant can be noted due to different expression control sequences being active in different tissues at different times, particularly in response to environmental factors and conditions.

II METHOD FOR TRANSFORMING A PLANT CELL USING HOMOLOGOUS RECOMBINATION

The present invention also includes a method for transforming a plant cell using homologous recombination. Preferably, the plant cell to be transformed has a nucleic acid sequence that does not naturally occur in the plant cell that can be expressed by the plant cell, such as a reporter gene. A nucleic acid sequence of interest can be targeted to integrate into the locus of such nucleic acid sequence that does not naturally occur in the plant cell using homologous recombination. The nucleic acid sequence of interest can include any gene, but preferably includes a bioremediation gene.

A cell of the present invention that expresses a gene of interest, such as a reporter gene, such as GUS, can be used as the basis of another aspect of the present invention. Cells of the present invention that express a gene of interest, such as a reporter gene, preferably wherein the

gene of interest is integrated into the genome of the cell, report that the reporter gene is being expressed. Homologous recombination can be used in order to insert another gene of interest, such as a bioremediation gene, at the locus of the reporter gene. In practice, a gene of interest is flanked with nucleic acid sequences that target the reporter gene and/or expression control sequences linked thereto, if they are present. This construct can form a vector, which is then used to transform the cell that is expressing the reporter gene. The vector with the gene of interest will, on occasion, insert by homologous recombination at the site of the reporter gene. Preferably, the inserted gene of interest will insert in-frame, will be expressed, and the expression product will have the desired biological activity.

The homologous recombination event will preferably knock-out the activity of the reporter gene. Thus, cells that lose reporter gene activity will presumptively have undergone homologous recombination at the site of the reporter gene. This event can be screened for using a variety of methods, including those described herein. The expression of the gene of interest in the cells can be determined by methods known in the art, such as Northern blot analysis to detect mRNA corresponding to the gene of interest and screening for the activity associated with the gene of interest. In the alternative, the reporter gene will not be knocked out, and the cells can be screened for the activity associated with the gene of interest. Furthermore, the vector used for the homologous recombination can include a selectable marker, such as antibiotic resistance. Cells that express the selectable marker would be protected from the antibiotic. Populations of cells that survive exposure to the antibiotic can then be screened for the activity associated with the gene of interest.

In this way, a cell made by the method of the present invention can be used to make a wide variety of cells that express a variety of genes of interest, such as one or more bioremediation genes. These cells can be cultured to form populations of cells, including callus. Plants can be regenerated from these cells using a variety of methods, including those described herein. Plants and seeds from these regenerated plants can also be obtained. Such cells, population of cells, callus, somatic embryos, immature inflorescence, plants and seeds are part of the present invention.

III METHOD FOR REGENERATING A PLANT - A

The present invention includes a method for regenerating a plant, such as a freshwater monocot plant, preferably a *Carex*, *Scirpus*, *Juncus* or *Typha*, preferably *Juncus effusus*, *Carex lurida* or *Scirpus polyphyllus*. The method includes provided a sample of a plant, inducing shoot development from the sample, and inducing root development from the sample. This aspect of the present invention includes plants made by this method and seeds derived therefrom.

Plants

The plant is preferably a freshwater monocot plant, a freshwater wetland monocot plant or a freshwater wetland monocot plant is a freshwater emergent wetland monocot plant. The plant is preferably a member of the genus *Carex*, *Scirpus*, *Juncus* or *Typha* and is preferably *Juncus effusus*, *Carex lurida* or *Scirpus polyphyllus*.

Sample of Plant

The sample of plant used in this method can be a cell, a population of cells, a portion of a plant, a callus, an embryo, an immature inflorescence, a seedling, an *in vitro* germinated seedling or any other sample of a plant that includes at least one viable plant cell. The sample preferably is derived from a transgenic plant cell, and is most preferably is derived from a transgenic plant made by a method of the present invention.

Inducing Shoot Development

Shoot development in the sample of the plant is preferably accomplished using culturing methods and medium that utilize plant growth regulators. The sample can be cultured on any appropriate medium, preferably Murashige and Skoog inorganic salts medium (MS) at full strength, supplemented with between about 10 grams/L and about 40 grams/L of sucrose. The media preferably includes between about 0.1 mg/L and about 100 mg/L, more preferably between about 1 mg/L and about 10 mg/L of at least one plant growth regulator (if more than one plant growth regulator is used, then the preferred ranges indicate the total concentration of the plant growth regulators). The plant growth regulator is preferably a cytokinin. The cytokinin is preferably one or more of the following: N6-benzyladenic (BA), N6-(2-isopentenyl)-adenine (2iP) and 6-furfurylaminopurine (kinetin). The sample in or on this medium is cultured at an appropriate temperature in the presence or absence of light, or a combination thereof. In this aspect of the invention, the samples are preferably cultured under continuous illumination until

shoot development is noted. Shoot development can take between about 10 days and about 8 weeks, preferably between about 2 weeks and about 4 weeks.

Inducing Root Development

Root development in the sample of the plant is preferably accomplished using culturing methods and medium that utilize plant growth regulators. The sample can be cultured on any appropriate medium, preferably Murashige and Skoog inorganic salts medium (MS) at full strength, supplemented with between about 10 grams/L and about 40 grams/L of sucrose. The media preferably includes between about 0.01 mg/L and about 50 mg/L, more preferably between about 0.1 mg/L and about 5 mg/L of at least one plant growth regulator (if more than one plant growth regulator is used, then the preferred ranges indicate the total concentration of the plant growth regulators). The plant growth regulator is preferably an auxin. The auxin is preferably one or more of the following: naphthaleanic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 3,6-dichloro-0-anisic acid (dicamba) and 4-amino-3,5,6-trichloropicolinic acid (picloram).

The medium preferably includes one or more of the following. Between about 0.1% and about 10%, preferably between about 0.5% and about 2% of powdered charcoal. Between about 0.5 milligrams/L and about 500 milligrams/L, preferably between about 5 milligrams/L and about 100 milligrams/L, most preferably between about 30 milligrams/L and about 60 milligrams/L of citric acid. Between about 0.5 milligrams/L and about 500 milligrams/L, preferably between about 5 milligrams/L and about 100 milligrams/L, most preferably between about 30 milligrams/L and about 60 milligrams/L of ascorbic acid.

The sample in or on this medium is cultured at an appropriate temperature in the presence or absence of light, or a combination thereof. In this aspect of the invention, the samples are preferably cultured in continuous light until root development is noted. Root development can take between about 5 days and about 8 weeks (**FIG. 2**).

Cultivating plants and seeds

The rooted plants are then transferred to cultivation media, such as natural or artificial potting soil in appropriate containers. The potted plants are cultivated under appropriate conditions of temperature, humidity, light and other factors associated with a particular plant. Mature plants can produce seeds. These plants and seeds are also aspects of the present invention.

IV METHOD FOR REGENERATING A PLANT - B

The present invention also includes a method for regenerating a plant, such as a freshwater monocot plant, preferably a *Carex*, *Scirpus*, *Juncus* or *Typha*, preferably *Typha latifolia* or *Typha angustifolia*. The method includes providing a sample of a plant, forming a callus from the sample, inducing shoot development and inducing root development from the sample. This aspect of the present invention includes plants made by this method and seeds derived therefrom.

Plants

The plant is preferably a freshwater monocot plant, a freshwater wetland monocot plant or a freshwater wetland monocot plant is a freshwater emergent wetland monocot plant. The plant is preferably a member of the genus *Carex*, *Scirpus*, *Juncus* or *Typha* and is preferably *Typha latifolia*.

Sample of Plant

The sample of plant used in this method can be a cell, a population of cells, a portion of a plant, a callus, an embryo, an immature inflorescence, a seedling, *in vitro* germinated seedling or any other sample of a plant that includes at least one viable plant cell. The sample preferably is derived from a transgenic plant cell, and is most preferably is derived from a transgenic plant made by a method of the present invention.

Forming a Callus

Callus formation from the sample of the plant is preferably accomplished using culturing methods and medium that utilize plant growth regulators. The sample can be cultured on any appropriate medium, preferably Murashige and Skoog inorganic salts medium (MS) at full strength, supplemented with between about 10 grams/L and about 40 grams/L sucrose and vitamins (thiamine (B1), nicotinic acid (B3) and pyridoxine (B6)). The media preferably includes between about 0.01 mg/L and about 50 mg/L, more preferably between about 0.1 mg/L and about 5 mg/L of at least one plant growth regulator (if more than one plant growth regulator is used, then the preferred ranges indicate the total concentration of the plant growth regulators). The plant growth regulator is preferably an auxin. The auxin is preferably one or more of the following: naphthaleanic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 3,6-dichloro-0-anisic acid (dicamba) or 4-amino-3,5,6-trichloropicolinic acid (picloram).

The sample in or on this medium is cultured at an appropriate temperature in the presence or absence of light, or a combination thereof. In this aspect of the invention, the samples are preferably cultured in continuous darkness until callus develops, which can take between about 1 week and about 9 weeks (**FIG. 3** through **FIG. 6**).

Inducing Shoot Development and Inducing Root Development

Shoot development and root development in the sample of the plant is preferably accomplished using culturing methods and medium that utilize plant growth regulators. The sample can be cultured on any appropriate medium, preferably Murashige and Skoog inorganic salts medium (MS) at full strength, supplemented with between about 10 grams/L and about 40 grams/L sucrose. The media preferably includes between about 0.1 mg/L and about 100 mg/L, more preferably between about 1 mg/L and about 10 mg/L of at least one plant growth regulator (if more than one plant growth regulator is used, then the preferred ranges indicate the total concentration of the plant growth regulators). The plant growth regulator is preferably a cytokinin. The cytokinin is preferably one or more of the following: N6-benzyladenic (BA), N6-(2-isopentenyl)-adenine (2iP) and 6-furfurylaminopurine (kinetin). The sample in or on this medium is cultured at an appropriate temperature in the presence or absence of light, or a combination thereof. In this aspect of the invention, the samples are preferably cultured in continuous darkness until shoot development and root development is noted. Shoot development can take between about 10 days and about 8 weeks. Root development can take between about 5 days and about 8 weeks (**FIG. 3**).

Cultivating Plants and Seeds

The rooted plants are then transferred to cultivation media, such as natural or artificial potting soil in appropriate containers. The potted plants are cultivated under appropriate conditions of temperature, humidity, light and other factors associated with a particular plant. Mature plants can produce seeds. These plants and seeds are also aspects of the present invention.

V METHOD FOR REGENERATING A PLANT - C

The present invention includes a method for regenerating a plant, such as a freshwater monocot plant, preferably a *Carex*, *Scirpus*, *Juncus* or *Typha*, preferably *Juncus accuminatus*. The method includes providing a sample of a plant, forming a callus from said sample, inducing shoot

development from the sample and inducing root development from the sample. This aspect of the present invention includes plants made by this method and seeds derived therefrom.

Plants

The plant is preferably a freshwater monocot plant, a freshwater wetland monocot plant or a freshwater wetland monocot plant is a freshwater emergent wetland monocot plant. The plant is preferably a member of the genus *Carex*, *Scirpus*, *Juncus* or *Typha* and is preferably *Juncus* *acuminatus* or *Typha* *angustifolia*.

Sample of Plant

The sample of plant used in this method can be a cell, a population of cells, a portion of a plant, a callus, an embryo, an immature inflorescence, a seedling, *in vitro* germinated seedling or any other sample of a plant that includes at least one viable plant cell. The sample preferably is derived from a transgenic plant cell, and is most preferably is derived from a transgenic plant made by a method of the present invention.

Forming a Callus

Callus formation from the sample of the plant is preferably accomplished using culturing methods and medium that utilize plant growth regulators. The sample can be cultured on any appropriate medium, preferably Murashige and Skoog inorganic salts medium (MS) at full strength, supplemented with between about 10 grams/L and about 40 grams/L sucrose and vitamins (B1, B3 and B6). The media preferably includes between about 0.01 mg/L and about 50 mg/L, more preferably between about 0.1 mg/L and about 5 mg/L of at least one plant growth regulator (if more than one plant growth regulator is used, then the preferred ranges indicate the total concentration of the plant growth regulators). The plant growth regulator is preferably an auxin. The auxin is preferably one or more of the following: naphthaleanic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 3,6-dichloro-0-anisic acid (dicamba) and 4-amino-3,5,6-trichloropicolinic acid (picloram).

The sample in or on this medium is cultured at an appropriate temperature in the presence or absence of light, or a combination thereof. In this aspect of the invention, the samples are preferably cultured in continuous darkness until callus develops, which can take between about 1 week and about 4 weeks.

Inducing Shoot Development

Shoot development in the sample of the plant is preferably accomplished using culturing methods and medium that utilize plant growth regulators. The sample can be cultured on any appropriate medium, preferably Murashige and Skoog inorganic salts medium (MS) at full strength, supplemented with between about 10 grams/L and about 40 grams/L sucrose. The media preferably includes between about 0.1 mg/L and about 100 mg/L, more preferably between about 1 mg/L and about 10 mg/L of at least one plant growth regulator (if more than one plant growth regulator is used, then the preferred ranges indicate the total concentration of the plant growth regulators). The plant growth regulator is preferably a cytokinin. The cytokinin is preferably one or more of the following: N6-benzyladenic (BA), N6-(2-isopentenyl)-adenine (2iP) and 6-furfurylaminopurine (kinetin). The sample in or on this medium is cultured at an appropriate temperature in the presence or absence of light, or a combination thereof. In this aspect of the invention, the samples are preferably cultured in continuous illumination until shoot development is noted. Shoot development can take between about 1 week and about 4 weeks.

Inducing Root Development

Root development in the sample of the plant is preferably accomplished using culturing methods and medium that utilize plant growth regulators. The sample can be cultured on any appropriate medium, preferably Murashige and Skoog inorganic salts medium (MS) at full strength, supplemented with between about 10 grams/L and about 40 grams/L sucrose. The media preferably includes between about 0.01 mg/L and about 50 mg/L, more preferably between about 0.1 mg/L and about 5 mg/L of at least one plant growth regulator (if more than one plant growth regulator is used, then the preferred ranges indicate the total concentration of the plant growth regulators). The plant growth regulator is preferably an auxin. The auxin is preferably one or more of the following: naphthaleanic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 3,6-dichloro-0-anisic acid (dicamba) and 4-amino-3,5,6-trichloropicolinic acid (picloram).

The sample in or on this medium is cultured at an appropriate temperature in the presence or absence of light, or a combination thereof. In this aspect of the invention, the samples are preferably cultured in continuous illumination until root development is noted. Root development can take between about 5 days and about 4 weeks.

Cultivating, plants and seeds

The rooted plants are then transferred to cultivation media, such as natural or artificial potting soil in appropriate containers. The potted plants are cultivated under appropriate conditions of temperature, humidity, light and other factors associated with a particular plant. Mature plants can produce seeds. These plants and seeds are also aspects of the present invention.

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VI METHOD FOR REGENERATING A PLANT - D

The present invention includes a method for regenerating a plant or forming a somatic embryo from a plant, such as a freshwater monocot plant, preferably a *Carex*, *Scirpus*, *Juncus* or *Typha*, preferably *Typha angustifolia*. The method includes providing a sample of a plant, forming a callus from said sample and inducing the formation of at least one embryo. This aspect of the present invention includes plants made by this method and seeds derived therefrom.

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Plants

The plant is preferably a freshwater monocot plant, a freshwater wetland monocot plant or a freshwater wetland monocot plant is a freshwater emergent wetland monocot plant. The plant is preferably a member of the genus *Carex*, *Scirpus*, *Juncus* or *Typha* and is preferably *Typha angustifolia*.

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Sample of Plant

The sample of plant used in this method can be a cell, a population of cells, a portion of a plant, a callus, an embryo, an immature inflorescence, a seedling, *in vitro* germinated seedling or any other sample of a plant that includes at least one viable plant cell. The sample preferably is derived from a transgenic plant cell, and is most preferably is derived from a transgenic plant made by a method of the present invention.

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Forming a Callus

Callus formation from the sample of the plant is preferably accomplished using culturing methods and medium that utilize plant growth regulators. The sample can be cultured on any appropriate medium, preferably Murashige and Skoog inorganic salts medium (MS) at full strength, supplemented with between about 10 grams/L and about 40 grams/L sucrose and optionally vitamins (B1, B3 and B6). The media preferably includes between about 0.01 mg/L and about 50 mg/L, more preferably between about 0.1 mg/L and about 5 mg/L of at least one

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FOOTNOTES

plant growth regulator (if more than one plant growth regulator is used, then the preferred ranges indicate the total concentration of the plant growth regulators). The plant growth regulator is preferably an auxin. The auxin is preferably one or more of the following: naphthaleanic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 3-6-dichloro-0-anisic acid (dicamba) and 4-amino-3,5,6-trichloropicolinic acid (picloram).

5 The sample in or on this medium is cultured at an appropriate temperature in the presence or absence of light, or a combination thereof. In this aspect of the invention, the samples are preferably cultured in continuous darkness or continuous light until callus develops, which can take between about 1 week and about 4 weeks.

Inducing Formation of Embryos

10 Somatic embryogenesis from the callus is preferably accomplished using culturing methods and medium that utilize plant growth regulators. The sample can be cultured on any appropriate medium, preferably Murashige and Skoog inorganic salts medium (MS) at full strength, supplemented with between about 10 grams/L and about 40 grams/L sucrose. The media preferably includes between about 0.1 mg/L and about 100 mg/L, more preferably between about 15 1 mg/L and about 10 mg/L of at least one plant growth regulator (if more than one plant growth regulator is used, then the preferred ranges indicate the total concentration of the plant growth regulators). The plant growth regulator is preferably a cytokinin. The cytokinin is preferably one or more of the following: N6-benzyladenic (BA), N6-(2-isopentenyl)-adenine (2iP) and 6-furfurylaminopurine (kinetin), preferably BA. The sample in or on this medium is cultured at an appropriate temperature in the presence or absence of light, or a combination thereof. In this aspect of the invention, the samples are preferably cultured in continuous illumination until embryogenesis is noted, which can take between about 1 week and about 4 weeks.

Cultivating plants and seeds

20 The somatic embryos are then transferred to cultivation media for germination, such as natural or artificial potting soil in appropriate containers. The potted plants are cultivated under appropriate conditions of temperature, humidity, light and other factors associated with a particular plant. Mature plants can produce seeds. These plants and seeds are also aspects of the present invention.

VII METHOD OF BIOREMEDIATION

The present invention also includes a method of bioremediation by exposing a plant made by a method of the present invention to an environment or sample that contains or is suspected of containing at least one contaminant whose concentration in the environment or sample can be reduced by the plant by, for example, uptake, sequestering, or volatilisation by said plant. In this instance, a plant can reduce the concentration of at least one contaminant by removing the at least one contaminant by, for example, volatilization, or can concentrate the at least one contaminant within, on or around the plant by, for example, uptake, concentration or sequestering of the at least one contaminant.

One aspect of this method of bioremediation includes: providing a plant made by a method of the present invention, and exposing said plant to an environment containing or suspected of containing at least one contaminant that can be uptaken, sequestered, volatilized or reduced by said plant; wherein the amount of said at least one contaminant in said environment is reduced.

A second aspect of this method of bioremediation includes: providing a plant made by the method of the present invention; exposing said plant to a sample containing or suspected of containing at least one contaminant that can be uptaken, sequestered, volatilized or reduced by said plant; wherein the amount of said at least one contaminant in said sample is reduced by said plant.

In these aspects of the present invention, plants of the present invention are exposed to an environment such as a local environment (for example, water, sediment, soil or the like) or sample containing or suspected of containing at least one contaminant, whereby the plant can cause a decrease in the amount of the contaminant in the environment. The plant and the environment should be chosen so that one suits the other. For example, if the environment is a wetland, then a wetland plant should be chosen. The use of perennial and annuals is a matter of choice to the skilled artisan. Also, the type of climate should be considered. For example, certain plants cannot tolerate harsh winters and may not survive year-to-year. This characteristic may be desirable or undesirable, depending on the particular circumstances. For example, it may be advantageous for a plant of the present invention not to survive from year-to-year.

Preferably, the at least one contaminant is in the soil, sediment or water, or any combination thereof. Under these circumstances, the bioremediation gene in the plants can be in any part of the plant, but preferably localize in the roots of the plant.

Depending on the type of bioremediation gene in the plant, the plant can accumulate or transform a contaminant. If the plant accumulates a contaminant, then after an appropriate period of time the plant is removed to remove the contaminant for the site or the sample. If the plant transforms the contaminant, such as in the case of mercury mer genes, then the plant need not be removed from the site or sample to remove the contamination from the site or sample.

The plant can be placed in a natural environment, or can be placed in an artificial environment. An artificial environment includes an environment modified such that the plant can perform its intended function. An environment can be changed by altering the terrain of the land to improve or modify, for example, water flow or drainage. An artificial environment can also be a separate from the environment, such as in a greenhouse or other structure or location. For example, dredgings or soil from contaminated sites can be moved to an artificial environment or a modified environment where at least one plant of the present invention can be grown such that at least one contaminant therein is reduced.

The ability of a plant and method of the present invention to reduce the amount of at least one contaminant in an environment or sample can be determined by measuring the amount of contaminants in an environment or sample over time in the presence and absence of the plant.

EXAMPLES

EXAMPLE 1: SEED ISOLATION AND GERMINATION

This example establishes methods for obtaining seeds and initiating germination thereof. *Typha latifolia* (Broadleaf Cattail) and *Typha angustifolia* (Narrowleaf Cattail) seeds were separated from the fruiting spikes by blending with water in a Waring blender. The blended mixture was poured into a water filled glass tank. The seeds settled to the bottom and were collected using a pipette. *Juncus accuminatus* (Bulrush), *Juncus effusus* (Softrush) and *Scirpus polyphyllus* (Many-leaved Bulrush) seed were separated from the dried inflorescence by tapping the inflorescence. The seeds were separated from the husk by passing through a metal screen

with a mesh of one square millimeter. For *Carex lurida* (Lurid Sedge) the seeds were dehusked by crushing the seeds gently in a mortar and pestle for thirty seconds, followed by rubbing the mixture between the hand palms for five seconds. The seeds were separated from the husks by shaking the mixture on a piece of paper so that the seeds rolled off.

Seeds were surface sterilized by stirring in water containing 0.4% Tween-20 for five minutes followed by thirty minutes in a 30% solution of bleach and 0.1% Tween-20. The seeds were rinsed with sterile water and transferred to sterile conical flasks completely filled with liquid MS basal medium containing vitamins and 3% sucrose (Murashige and Skoog, 1962). Seeds were incubated under continuous light to induce germination (30 micromol / m² s, 25° C). Some seeds required 45% bleach for thirty-five minutes, optionally with the addition of a five minute immersion in a 0.1% solution of mercuric chloride for disinfestation.

EXAMPLE 2: CULTURE INITIATION, CULTURE REGENERATION, MEDIA, AND GREENHOUSE AND WETLAND ESTABLISHMENT

This example establishes culture and greenhouse methods to obtain plant materials for genetic manipulation. The seeds obtained in Example 1 were *in vitro* germinated to seedlings measuring between five and ten millimeters in length. Seedlings reached this size in three days for *Typha* and six to eight days for the other plants. Seedlings were cultured on gelled callus induction medium, which was basal medium supplemented with one of the auxins NAA, 2,4-D, dicamba or picloram (each at 1, 2, 5, and 10 milligrams/L). The effect of pH on these seedlings was evaluated by adjusting the medium to various pH levels and observing the effects thereof. For callus induction, *J. accuminatus* explants were cultured for three weeks and *Typha* for nine weeks in the dark. Embryogenic structures were stained for the presence of starch using an iodine-potassium iodide (I₂ - KI) solution.

For shoot induction in *Typha* and *J. accuminatus* calli were transferred to gelled basal medium supplemented with 1.5 or 10 milligrams/L of N⁶-Benzyladenine (BA). In *J. accuminatus* shoots were rooted by culturing in Magenta boxes containing fifty milliliters of gelled medium supplemented with 0.1 milligrams/L of NAA. *Typha* shoots were rooted on shoot induction medium. Shoot and root induction were carried out under continuous illumination under the conditions set forth in Example 1 for germination.

Callus of certain plants tends to brown and become undesirable for later protocols. Thus, regeneration protocols for *J. effusus*, *C. lurida* and *S. polyphyllus* did not include a callus phase. Seedling were cultured directly on gelled basal medium supplemented with BA, 2iP or kinetin (each at 1, 5, and 10 milligrams/L) and cultured in the light for shoot development. After six weeks on 2iP medium, multiple shoots of about 1 to 1.5 centimeters in length were separated for rooting. *C. lurida* and *S. polyphyllus* shoots were rooted in the same rooting medium used for *J. accuminatus*. Due to shoot browning, *J. effusus* shoots were transferred to boxes containing fifty milliliters of one of three rooting media: 1) MS + NAA (1 milligram/L), 2) MS + NAA (1 milligram/L) + charcoal (1% w/v), and 3) MS + NAA (1 milligram/L) + citric acid (50 milligrams/L) + ascorbic acid (100 milligrams/L).

After nine weeks for *Typha* and about four to five weeks for other plants, plantlets were removed from the media, the roots washed to remove the media, and the plantlets potted into 1.5 in square pots containing commercially available potting soil. Plants were covered with transparent plastic for two weeks to reduce water loss, and placed in a greenhouse with natural light intensity (230 micromol/m² s).

Callus induction from *Typha* leaves, roots and rhizomes from mature plants was problematic due to systemic contamination of these tissues. The use of *in vitro* germinated seeds proved superior and provide a year-round source of materials.

Results

For callus cultures of *T. latifolia*, *T. angustifolia* and *J. accuminatus* picloram, 2,4-D and NAA induced callus growth. Picloram at 5 milligrams/L was preferable. In both species, callus proliferated mainly from the root-shoot junction. BA at a concentration of 5 milligrams/L in B5 medium induced callus shoot regeneration by organogenesis. In *T. latifolia* and *T. angustifolia*, 37 % and 55%, respectively, of the explants regenerated shoots, with *T. latifolia* explants forming twice as many shoots as did *T. angustifolia* explants. For *J. accuminatus*, callus age (up to 50 weeks) did not influence regeneration ability.

The ability to withstand extremes of pH is a practical trait in wetland. Effect of medium pH (3.8 to 7.8) callus age (3, 5, and 7 weeks) and callus environment (dark or continuous light) on *J. accuminatus* regeneration were determined for *J. accuminatus*. All the parameters significantly influenced regeneration. Callus induction and shoot regeneration occurred on B5

media ranging in pH from 3.8 to 7.8, showing its adaptability to a wide range of pH *in vitro* (TABLE 1). Regenerated shoots were multiplied by subculture onto B5 medium at four week intervals. The regenerated shoots rooted within a week on 0.1 milligrams/L of NAA supplemented medium. Upon transfer to the greenhouse the plants had a greater than 95% survival level, and flowered and set viable seed after three months in the greenhouse. Plants covered with plastic grew faster and flowered earlier, than uncovered plants. No albinos or abnormal plants have been observed when these methods have been used.

TABLE 1: Effect of media pH on callus growth, frequency of shoot regeneration, and number of shoots per calli of *Juncus accuminatus*. Means in the columns followed with the same letter are not significantly different at 5% level using Duncan's multiple range test (NS = nonsignificant).

Media pH before autoclaving	Callus dry weight (milligrams)	Percent callus forming shoots	Number of shoots per regenerating callus
7.8	1.8 b	33 a	2.0 b
5.8	2.0 a	55 a	3.6 a
3.8	1.6 c	40 a	2.0 b
ANOVA			
Medium pH	P < 0.05	NS	P < 0.05

T. latifolia shoots rooted by nine weeks on shoot induction medium, as has been reported in *Distichlis* (Straub et al. (1989)). *T. angustifolia* callus cultured on picloram, 2,4-D or dicamba, in the dark or in the light, proliferated embryos within about 5 to 10 weeks. Twenty percent of cultures on dicamba (2 milligrams/L) produced somatic embryos within two months. The embryos were starch rich, globular and bipolar. Culturing the embryos on B5 medium promoted germination within two to four weeks, whereas *Phragmites* embryos germinate on medium without growth regulators (Straub et al., 1988). The rooted plants were acclimatized to greenhouse conditions.

J. effusus callus upon transfer to cytokinin containing medium, in the light, can turn black within seventy-two hours. This type of blacking has been reported in *Distichlis* callus (Straub et

al. (1989)). *J. effusus* could be regenerated, however, by culturing seedling explants directly on cytokinin media in the light. The optimal preferred concentration of cytokinins was about 5 milligrams/L. 2iP caused the highest regeneration frequency and number of shoots per explant. Kinetin caused the poorest response. A range of medium pH (about 3.8 to about 7.8) was tested for effects on shoot proliferation. At all medium pH's, shoots were induced (TABLE 2). The shoot proliferation did not differ significantly at media pH values between about 3.8 and about 5.8 in the presence of 2iP and BA. Proliferation was low at pH 7.8 regardless of cytokinin used. Such an effect of pH on direct shoot induction has not been reported for any other wetland species. The ability of some wetland plants to tolerate a wide range of soil pH may reflect this tolerance in vitro (Guntenspergen et al. (1989)). The in vitro generated shoots, upon additional subculture to B5 medium, produced higher numbers of shoots, compared to seedling explants. The regenerated shoots were brown at the base and gradually turned dark. Antioxidants and activated charcoal were used to prevent browning/blackening in vitro (Jones and Clayton-Greene (1992); and Fridborg et al. (1978)). Charcoal in the rooting medium increased root and shoot growth and decreased rooting time, whereas addition of the antioxidants citric acid and ascorbic acid did not (TABLE 3). The rooted plants were acclimatized to the greenhouse with a survival rate of more than 95%.

TABLE 2: Effect of cytokinins and pH on *Juncus effusus* seedling explants regeneration. Means in columns and rows not followed by the same letter are significantly different at the 5% level using Duncan's Multiple Range Test. Upper case letters are for row comparison and lower case letters are for column comparison.

Cytokinin	Media pH		
(22.2 microM)	3.8	5.8	7.8
PERCENT EXPLANTS REGENERATING SHOOTS			
2iP	89 Aa	86 Aa	70 Ba
BA	84 Aa	88 Aa	46 Bb
Kinetin	26 Bb	36 Ab	27 Bc
SHOOT NUMBER PER RESPONDING EXPLANT			
2iP	5.5 Aa	5.8 Aa	4.3 Ba
BA	3.0 Bb	3.5 Ab	1.9 Cb
Kinetin	2.4 Ab	2.6 Ac	1.4 Bc

TABLE 3: Effects of rooting medium additives on in vitro growth of *Juncus effusus*. \pm Standard deviation. Whole plant (mg) is in fresh weight.

Parameters	Additives		
	None	Ascorbic Acid and Citric Acid	Charcoal
Days for Rooting	32.2 ± 2.0	28.0 ± 2.7	17.1 ± 3.0
Root Length (cm)	2.5 ± 0.5	2.5 ± 0.7	23.1 ± 1.3
Shoot Length (cm)	5.5 ± 0.5	7.8 ± 0.6	10.5 ± 0.7
Whole Plant (mg)	263.7 ± 42.5	392.2 ± 63.0	671.0 ± 60.4

Three to six-month-old greenhouse established plants, grown in pots ranging in size from 3 to 8 inches in diameter, of *Typha latifolia*, *T. angustifolia*, *Juncus accuminatus*, *J. effusus*, *Scirpus polyphyllus* and *Carex lurida* were planted in a freshwater constructed wetland habitat in the early spring (**FIG. 7**, **FIG. 8** and **FIG. 9**).

The constructed wetland is located in north central West Virginia (WV). It is an area where freshwater drainage from a natural watershed collected. The area is periodically or intermittently flooded with shallow water. It supports native emergent and submerged herbaceous plants including, but not limited to, *Typha* and *Juncus*. Some of the plants like *T. latifolia* were established from rhizomes dug from a natural wetland and transplanted in the site. Some plants established naturally from wind, water or animal born seed or other propagules.

The plants were planted at the average date when the area was frost-free (in WV they were planted May 1). Holes 2 to 3 inches larger than the size of the pot each plant was grown in were dug in an area of the wetland containing shallow standing water (at that time of year). The plants were removed from the pots and placed in the water filled holes, with the level of the plant in the pot set level with the soil bottom of the flooded area.

J. effusus was also planted in an open grassy terrestrial area in north-central WV. Holes 2 to 3 inches larger than the pot size were dug, and the plants set in the hole and the root ball covered with soil to the same level as was present in the potted plant. The plants were watered weekly, using a garden hose.

All plants rapidly became well established. All plants appeared normal, no variations in phenotype were observed in the established plants. The plants thrived and competed vigorously with naturally established *Juncus* and *Typha* already present in the constructed wetland. The *J.*

accuminatus plants, which had bloomed and set seed in the greenhouse, continued to do so in the wetland. The *J. effusus* plants, in both the wetland and terrestrial sites, bloomed and set seed within three months of transplanting outdoors.

EXAMPLE 3: ANTIBIOTICS, BACTERIA STRAINS, PLASMIDS, BOMBARDMENT, CULTURE CONDITIONS AND GUS ACTIVITY

5 This example establishes materials and methods for transfection and selection of plant materials obtained using the methods of Example 1 and Example 2. To determine the effects of antibiotics on plant materials, such as seedlings and callus, seedlings were cultured in the dark on callus induction medium containing five milligrams/L of picloram (P5 medium), supplemented with an antibiotic or mercuric chloride. In order to use *nptII* (neomycin phosphotransferase) gene, *hptII* (hygromycin phosphotransferase) gene and *merA* (modified bacterial mercuric reductase) gene as selectable markers, kill curves of non-transformed plant materials were performed with hygromycin, kanamycin and mercuric chloride. Such kill curves were performed using
10 hygromycin at 10, 20, 30, 40, 50, 60 and 75 milligrams/L; kanamycin at 25, 50, 75, 100, 150 and 200 milligrams/L; and mercuric chloride at 1.3, 2.7, 4.0, 5.4 and 6.7 milligrams/L. Cerbenicillin and cefotaxime were tested at 500 and 250 milligrams/L, respectively.

15 *Agrobacterium* EHA105 (Hood et al., 1986) harboring plasmid pBISN1 (FIG. 1) or pKiwi105 (Narasimhulu et al. (1996); and Janssen and Gardner (1989)). pBISN1 contains GUS whose expression is driven by the "Super Promoter" (a hybrid of *mas* and *ocs*) while pKiwi105 contains GUS whose expression is driven by *mas* and the 35S promoter. Callus was generated from seedlings cultured in the dark on P5 medium following the general procedures described in
20 these Examples. *Agrobacterium* was grown to 1 OD at 600 nm in LB medium containing 50 milligrams/L kanamycin at 28°C at 200 RPM shaking. Calli were immersed in the bacteria cultured for five to ten minutes, blotted on sterile filter paper, and cocultivated on P5 medium for two days. These calli were transferred to P5 medium containing 250 milligrams/L of cefotaxime. After culturing for one week in the dark, calli were stained for GUS activity generally following
25 established procedures (Jefferson et al. (1987)).

For bombardment procedures, a single layer of calli was deposited on filter paper. Particle bombardment (microballistics or ballistics) of the calli using pAL77 constructed in pFC3 (Lonsdale et al., 1995) and an ACT/2GUS construct (An et al., 1996) was performed using
30 established methods using a Bio-Rad PDS-1000/He particle gun (Rugh et al., (1998)). The

bombarded calli-filters were placed on P5 medium and cultured for one week in the dark and stained for GUS activity.

Results

Transient GUS expression studies showed that *Typha*, *Juncus*, *Carex* and *Scirpus* were transformed by *Agrobacterium*. GUS expression could be used to monitor transient gene expression and to evaluate conditions that enhance transformation systems (Hiei et al. (1994)).

In preliminary experiments the frequency of transient GUS activity was measured by scoring individual calli having one or more GUS positive sites. Relatively low frequencies of GUS expression (between about 1% and about 6% of calli) were obtained in all plants cocultivated with *Agrobacterium* containing pKiwi105 with the 35S and *mas* promoter. Young callus (about one month old) had a higher frequency of transient GUS expression than did older cultures. The highest frequency of transient GUS expression obtained was about 40% in embryonic cultures of *T. angustifolia* cocultivated with *Agrobacterium* containing pBISN1 with GUS expression driven by the "Super Promoter." The differences in expression may have been due to the promoter an/or the developmental state of the culture (embryogenic) as is seen in corn, although the inventors expressly do not wish to be limited to any proposed mode of action or mechanism (Schlappi and Hohn (1992)). Microprojectile bombardment transformation using promoters such as 35S, Ubi and ACT11 showed relatively low expression of GUS. Frequencies of expression with these promoters were relatively low, between about 1% and about 6%, which correlates with the results obtained from the *Agrobacterium* studies. To reduce the number of *Agrobacterium* in a culture, cefotaxime was found superior to carbenicillin as was observed in wheat (Mathias et al. (1986)). In this instance, carbenicillin induced root production. Mercuric chloride, kanamycin and hygromycin could be used to prevent the growth of *T. angustifolia*, *T. latifolia* and *J. accuminatus* and thus are potential selective agents for non-transformed cells (TABLE 4).

TABLE 4: Chemical inhibition of callus proliferation. Seedlings cultured six weeks in the dark with 5 milligrams/L of picloram with the indicated chemical. ND = Not Done, * = No Clear Cut Response.

Chemical	Concentration (mg/L) Resulting in Growth Inhibition		
	<i>T. angustifolia</i>	<i>T. latifolia</i>	<i>J. accuminatus</i>
Hygromycin	20	20	60
Kanamycin	200 *	200 *	ND
Mercuric chloride	2.7	2.7	5.4

All publications, including patent documents and scientific articles, referred to in this application and the bibliography and attachments are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication were individually incorporated by reference.

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